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INTERCOURSE BETWEEN CELL WALL AND CYTOPLASM EXEMPLIFIED BY ARABINO GALACTAN PROTEINS AND CORTICAL MICROTUBULES¹

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How does a plant cell sense and respond to the status of its cell wall? Intercourse between cell wall and cytoplasm has long been supposed to involve arabinogalactan proteins, in part because many of them are anchored to the plasma membrane. Disrupting arabinogalactan proteins has recently been shown to disrupt the array of cortical microtubules present just inside the plasma membrane, implying that microtubules and arabinogalactan proteins interact. In this article, we assess possibilities for how this interaction might be mediated. First, we consider microdomains in the plasma membrane (lipid rafts), which have been alleged to link internal and external regions of the plasma membrane; however, the characteristics and even the existence of these domains remains controversial. Next, we point out that disrupting the synthesis of cellulose also can disrupt microtubules and consider whether arabinogalactan proteins are part of a network linking microtubules and nascent microfilaments. Finally, we outline several signaling cascades that could transmit information from arabinogalactan proteins to microtubules through channels of cellular communication. These diverse possibilities highlight the work that remains to be done before we can understand how plant cells communicate across their membranes.

Key words: arabinogalactan protein; cell wall; cellular communication; cortical microtubules; morphogenesis; roots; signaling cascades.

The first thing we learn about plant cells is that they have a cell wall—a matrix dense with crosslinked polysaccharide and proteinaceous polymers. But plant cell walls cannot be viewed as dead chambers sheltering living monks, as imagined by Robert Hooke looking at cork; instead, the cell wall is part of the living cell and must connect to the cytoplasm intimately. The connection is reasonably viewed as mediated by plasma membrane receptors that bind relevant ligands of the cell wall (e.g., Hématy et al., 2007). But this view is incomplete: it places the cell wall beyond the cell as another part of the environment to be sensed, and it ignores information flow from cytoplasm to cell wall. Besides plasma-membrane receptors, we have only a limited understanding of how the cytoplasm and cell wall maintain their intimate, informational contact.

In this connection, intriguing components of the cell wall are arabinogalactan-proteins. Unlike most cell wall components, many of these glycoproteins are anchored to the plasma membrane, where they are plentiful. Thus, arabinogalactan proteins are positioned ideally to transmit information between cell wall

and cytoplasm; that they do so is supported by a growing body of evidence (Humphrey et al., 2007; Seifert and Roberts, 2007). Nevertheless, the way in which arabinogalactan proteins transmit information remains largely unknown, perhaps because the number of different arabinogalactan proteins is large and the structure of their polysaccharide moieties is complex.

A clue about this transmission is offered by recent observations that interfering with arabinogalactan proteins causes a concomitant disruption to the cortical array of microtubules (Sardar et al., 2006; Nguema-Ona et al., 2007). The joint disruption is unlikely to be explained by direct contact because, as far as is known, microtubules are cytosolic and arabinogalactan proteins are completely extracellular. Although arabinogalactan proteins might exist that span the membrane, we assume that microtubules and arabinogalactan proteins are linked indirectly. Here, we explore the interaction between arabinogalactan proteins and cortical microtubules, aiming to chart channels of communication across the plasma membrane.

Arabinogalactan proteins and cortical microtubules—Arabinogalactan proteins have been reviewed comprehensively (Fincher et al., 1983; Nothnagel, 1997; Schultz et al., 2000; Seifert and Roberts, 2007). Briefly, they consist of a polypeptide backbone decorated with arabinogalactan-rich glycans, which typically amount to more than 90% of the mass of the macromolecule. Many arabinogalactan-protein sequences contain a canonical signal for a glycosylphosphatidylinositol (GPI) anchor, an attachment that moors them on the external leaflet of the plasma membrane (Orlean and Menon, 2007). Nevertheless, these polymers are present throughout the cell wall and are even secreted into the medium of cultured cells, both because the GPI anchor is often cleaved, thereby releasing the proteoglycan

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into the wall and because some arabinogalactan proteins are synthesized without a membrane anchor.

Since the 1960s, it has been recognized that a characteristic feature of arabinogalactan proteins is their specific binding to certain phenylglycosides, termed Yariv reagents after their discoverer (Yariv et al., 1962). Those containing β -D-glucose or β -D-galactose bind arabinogalactan proteins with high affinity and will be referred to here as active Yariv, whereas phenylglycosides containing β -D-mannose or α -D-glucose bind with low affinity, if at all, and will be referred to here as inactive Yariv. Initially, active Yariv reagent was used to purify and quantify arabinogalactan proteins and to localize them within organs; however, the discovery in the 1990s that active Yariv disrupts growth has made it an invaluable probe for studying arabinogalactan protein function in vivo.

A pioneering demonstration of the biological effect of active Yariv reagent on plant development was the inhibition of proliferation of suspension-cultured rose cells (Serpe and Nothnagel, 1994). These authors suggested that cell division was inhibited because arabinogalactan proteins signal across the plasma membrane into the cell, speculating that the cross-linking of arabinogalactan proteins by active Yariv alters the alignment of cortical microtubules or impedes their depolymerization during prophase, thereby preventing mitosis. A difficulty with this idea is that blocking mitosis with microtubule inhibitors seldom inhibits expansion in cultures, leading instead to large, swollen cells (Weerdenburg and Seagull, 1988; Yoneda et al., 2007), whereas the rose cells treated with active Yariv stopped dividing and expanding (Serpe and Nothnagel, 1994). Nonetheless, a few years later, active Yariv was shown to cause epidermal cells in arabidopsis roots to bulge (Willats and Knox, 1996; Ding and Zhu, 1997), a phenotype that is consistent with microtubule dysfunction (Bannigan et al., 2006).

Additional evidence linking arabinogalactan proteins and cortical microtubules came from an arabidopsis mutant, *root epidermal bulger1 (reb1)* (Baskin et al., 1992). This mutant has a reduced elongation rate of its primary root and has swollen epidermal cells, which resemble a wild-type root treated with active Yariv. Roots of the mutant contain less arabinogalactan protein than those of the wild type (Ding and Zhu, 1997). Subsequently, Andème-Onzighi et al. (2002) showed that bulging of epidermal cells in *reb1* is restricted to trichoblasts and that the trichoblasts have abnormal cell wall ultrastructure and express certain arabinogalactan protein epitopes less abundantly (Fig. 1). These authors also found that cortical microtubules in trichoblasts are markedly disorganized and, in view of this, hypothesized that arabinogalactan proteins are required for cortical microtubule organization.

The identification and cloning of the *REB1* gene was reported in parallel (Seifert et al., 2002). The *REB1* locus encodes one of five UDP-D-glucose 4-epimerase isoforms (UGE4) involved in the synthesis of D-galactose. Interestingly, while the level of galactosylation of arabinogalactan proteins and xyloglucan is decreased in *reb1* trichoblasts, the level is unchanged in the pectic polysaccharides rhamnogalacturonan I and II (Nguema-Ona et al., 2006). This supports the suggestion that the epimerase isoforms participate in metabolic channeling of galactose into distinct cell wall polymers (Seifert, 2004).

In *reb1*, the disorganized microtubules and a lowered abundance (or perhaps aberrant carbohydrate structure) of arabinogalactan proteins were only correlated. To test causality, Sardar et al. (2006) examined microtubules in tobacco BY-2 cells treated with active Yariv for 5–24 h: along with cell swelling, they re-

ported microtubule disorganization. However, 5 h is a relatively long time. Treatment with active Yariv profoundly disrupts cell wall assembly in lily pollen tubes after 1 h (Roy et al., 1998) and in arabidopsis roots after 2 h (Nguema-Ona et al., 2007). Furthermore, treatment of lily pollen tubes and tobacco BY-2 cells with active Yariv causes, within minutes, a sustained increase in cytosolic calcium (Roy et al., 1999; Pickard and Fujiki, 2005). Either disrupted cell wall structure in general or elevated calcium in specific could have disrupted the microtubules.

Using arabidopsis roots, members of our laboratories showed that active Yariv caused significant microtubule disorganization, even within 15 min of application (Nguema-Ona et al., 2007; Fig. 2A, B). The microtubule disorganization was insensitive to treatment with gadolinium, a calcium channel blocker that successfully prevented microtubule depolymerization caused by exogenous calcium. Microtubules were disorganized by as low as 1 μ M active Yariv, not by inactive Yariv, and were also disorganized by treatment with monoclonal antisera specifically recognizing arabinogalactan protein epitopes (JIM13 and JIM14). Active Yariv as well as the antisera caused the arabinogalactan protein epitopes to aggregate within the plasma membrane. In addition, in a microscopical analysis of high-pressure frozen cells, active Yariv caused detachment of cortical microtubules from the plasma membrane (Fig. 2C, D) and aberrant cell wall deposition (Fig. 2E).

Taken together, the results of Andème-Onzighi et al. (2002), Sardar et al. (2006), and Nguema-Ona et al. (2007) establish an interaction between cortical microtubules and arabinogalactan proteins. What is the nature of this interaction? Does it reflect communication across the plasma membrane?

Rafting down the river—Communication between inside and outside leaflets of the plasma membrane has been suggested recently to be fostered by lipid rafts, hypothetical membrane domains that are rich in sterols and sphingolipids as well as certain types of proteins, including those with a GPI anchor (Mayor and Rao, 2004; Mukherjee and Maxfield, 2004; Simons and Vaz, 2004). By virtue of composition, raft lipids are said to form a lipid-ordered state, in contrast to the rest of the membrane, said to be in a lipid-disordered state. The ordered domain hypothetically traps proteins with appropriate anchors, including transmembrane proteins, and influences the lipids in both leaflets of the bilayer. The contents of lipid rafts have been inventoried by extracting plasma membranes at 4°C with 1% Triton-X100; the insoluble material (proteins and lipids), called the detergent-resistant membrane fraction, is defined as the lipid raft. In plants, the detergent-resistant fraction contains, among many proteins, tubulin and arabinogalactan proteins (Mongrand et al., 2004; Borner et al., 2005; Lefebvre et al., 2007), with the presence of the latter presumably accounted for by virtue of their GPI anchor.

Sardar et al. (2006) invoked lipid rafts to explain the connection between arabinogalactan proteins and microtubules. One may visualize an outer lipid-ordered domain, containing arabinogalactan proteins, and an inner domain juxtaposed to cortical microtubules, perhaps aided by membrane-binding or membrane-spanning, microtubule-associated proteins (Gardiner et al., 2001). Consistent with linkage by lipid rafts, another GPI-anchored protein, named COBRA, has been associated with cortical microtubules. Identified from a root morphology mutant (Benfey et al., 1993), COBRA has since been shown to be preferentially expressed in rapidly elongating cells, required for synthesis and orientation of cellulose microfibrils,

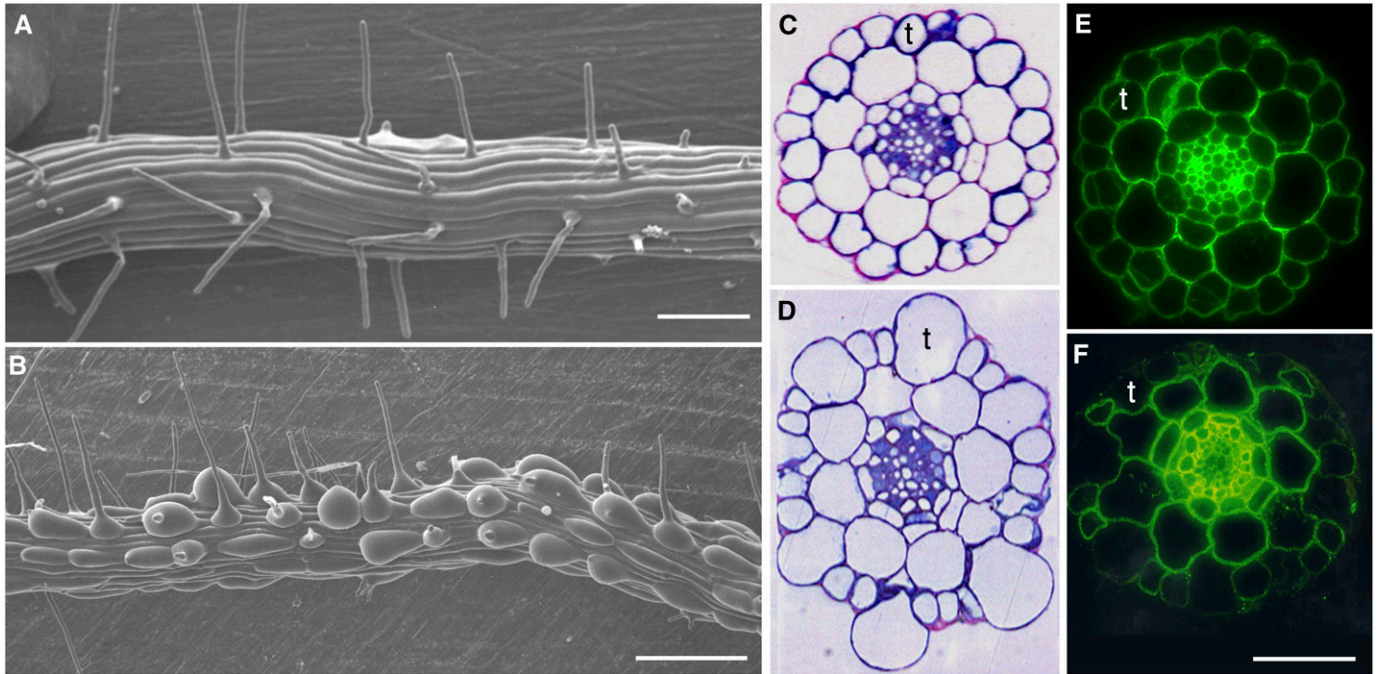


Fig. 1. Phenotype of root epidermal *bulger1-1* (*reb1-1*) of *Arabidopsis thaliana*. (A, B) Cryoscanning electron micrographs showing the surface of the root in (A) wild type and (B) *reb1-1*. Swollen cells are seen in trichoblast files. Not every trichoblast swells, and swellings are variable in size and in whether the swollen cell produces a root hair. (C, D) Toluidine-blue-stained cross-sections through the root elongation zone of (C) wild type and (D) *reb1-1*. In *arabidopsis*, trichoblasts (t) invariably form in those epidermal cells that contact the longitudinal-radial cell wall running between cortex cells. Some trichoblasts in the *reb1-1* section are swollen. (E, F) Cross-sections of (E) wild type and (F) *reb1-1* stained with monoclonal antibody JIM14, specific for arabinogalactan proteins. Note that in wild type, trichoblasts and atrichoblasts (i.e., epidermal cells adjacent to trichoblasts) are comparably stained, whereas in *reb1-1* trichoblast staining is weak or absent compared to that of the atrichoblasts. Trichoblasts are not swollen in (F) because the section was cut closer to the tip than where swelling occurs. Bars = 100 μ m (A), 250 μ m (B), and 50 μ m (C–F). Panels C–F were previously published as Figs. 2G, H and 4C, F in Andème-Onzighi, C., M. Sivaguru, J. Judy-March, T. I. Baskin, and A. Driouich (2002) The *reb1-1* mutation of *arabidopsis* alters the morphology of trichoblasts, the expression of arabinogalactan proteins and the organization of cortical microtubules. *Planta* 215: 949–958, and reproduced with kind permission from Springer Science & Business Media.

and, strikingly, localized in tracks overlying microtubules (Schindelman et al., 2001; Roudier et al., 2005).

While this image might be as idyllic as rafting down the Nile, the river has crocodiles. The nature and properties of lipid rafts remain the subjects of considerable controversy (Munro, 2003; Jacobson et al., 2007). Detergent extraction seems reasonable as a biochemical assay but not as a structural one. The cytoskeleton in nonmuscle cells was discovered on the basis of detergent insolubility (Brown et al., 1976), but the fibrous tangle remaining after extraction bears little relation to the fine, dynamic network of living cells. For membrane components, detergent resistance can indicate a shared biochemical property but cannot be taken as evidence that components so endowed aggregate into a separate phase in the absence of the detergent. Efforts to image lipid-raft domains in living cells produce widely divergent results (Jacobson et al., 2007) but commonly suggest extremely small and dynamic structures. For example, GPI-anchored proteins in various cultured vertebrate cell lines formed clusters of two to four protein molecules (Sharma et al., 2004). Therefore, while the compositional complexity of the plasma membrane plausibly supports distinct domains on the nano-scale, it is simplistic to think of them as stable platforms where interesting signaling molecules comfortably congregate.

Parallels with the microtubule–microfibril syndrome?—In considering how microtubules and arabinogalactan proteins

might communicate, it is instructive to consider attempts to link cortical microtubules to the plasma-membrane complex that synthesizes cellulose. Since their discovery, cortical microtubules have been suggested to orient the deposition of cellulose microfibrils (Ledbetter and Porter, 1963; Hepler and Newcomb, 1964) but even though cellulose synthase has cytosolic domains, direct connections between microtubules and subunits of the synthase remain elusive. As an alternative to a direct linkage, one of us has modeled the interaction between microtubules and microfibrils as involving the nascent microfibril rather than the cellulose synthase rosette (Baskin, 2001; Baskin et al., 2004). In this model, cortical microtubules coordinate a scaffold of proteins and lipids, some of which have extracellular domains that bind microfibrils (Fig. 3A). These cellulose-binding domains would be oriented ultimately with respect to the underlying microtubule, and they would bind the newly synthesized microfibril soon after it emerges from the rosette. The scaffold was suggested to include COBRA (Roudier et al., 2005) and could include arabinogalactan proteins.

Recently, the relation between microtubules and microfibril synthesis has been powerfully examined with live-cell imaging. In *arabidopsis* hypocotyls, fluorescently tagged cellulose synthase complexes (rosettes) have been observed moving in paths overlying cortical microtubules (Paredes et al., 2006). In further work with the tagged cellulose synthase, DeBolt et al. (2007) identified a coumarin derivative, named morlin, that causes

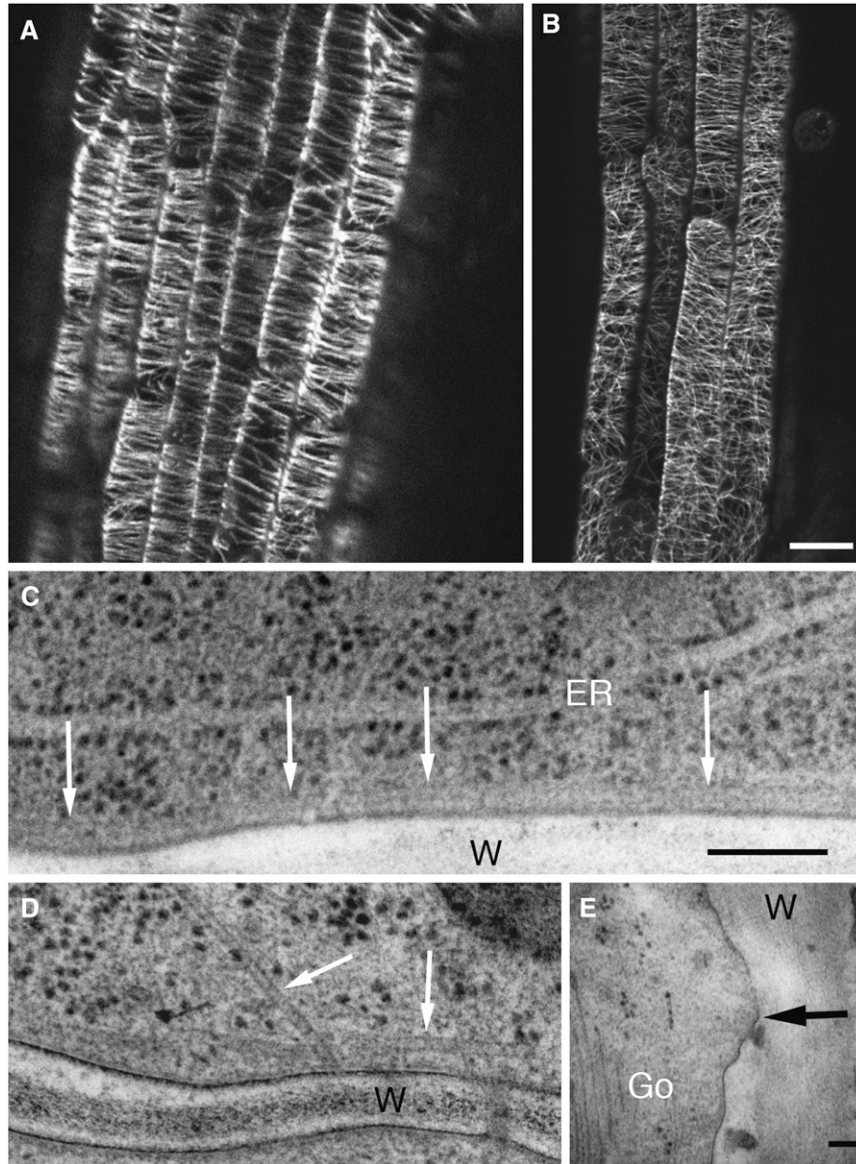


Fig. 2. Effect of active Yariv on cortical microtubule organization in *A. thaliana*. (A, B) Cortical microtubules imaged in living root epidermis expressing a green fluorescent protein (GFP)-microtubule reporter in (A) control and (B) a root treated with 5 μ M active Yariv for 22 min. (C–E) Electron micrographs showing the status of microtubules and cell wall in cryofixed epidermal cells in (C) control and (D, E) roots treated with 5 μ M active Yariv for 2 h. Cortical microtubules (white arrows) are up against the plasma membrane in control material (C), as usual for plant cells, whereas in Yariv-treated material (D), the microtubules are farther from the membrane, often reaching into the cell. In control material (C), the plasma membrane nearly always runs smoothly against the cell wall, which has a uniform texture, whereas in Yariv-treated material (E), the membrane often separates from the cell wall (black arrow), with amorphous deposits between the undulating membrane and parts of cell wall with uniform texture. W, cell wall; ER, endoplasmic reticulum; Go, Golgi stack. Bars = 10 μ m (A, B), 200 nm (C, D), 40 nm (E).

swollen morphology in seedlings and alters the behavior of both microtubules and rosettes. In morlin-treated hypocotyls, cortical microtubules are disorganized, their dynamics are suppressed, and in some backgrounds they become bundled and tend to dissociate from the plasma membrane; at the same time, morlin decreases the velocity of cellulose synthase movement and causes partial aggregation among rosettes, changes that morlin causes even in the absence of microtubules. DeBolt et al. (2007) hypothesize that morlin acts neither on microtubules nor synthase directly but instead targets a distinct protein that interacts with both. Another compound, cobtorin, causes cell swelling

and uncouples the orientations of microtubules and microfibrils but does not reduce cellulose synthesis or disorganize microtubules (Yoneda et al., 2007). The targets of morlin and cobtorin could be part of a protein–lipid scaffold that allows cortical microtubules and extracellular components to interact. Such a scaffold might include several arabinogalactan proteins, and if so, when these are aggregated, the concomitant disturbance to the scaffold could disrupt microtubule organization (Fig. 3B).

Doorbells and penny whistles: Kinases and lipases—Ideas like lipid rafts and scaffolds all reflect an underlying premise

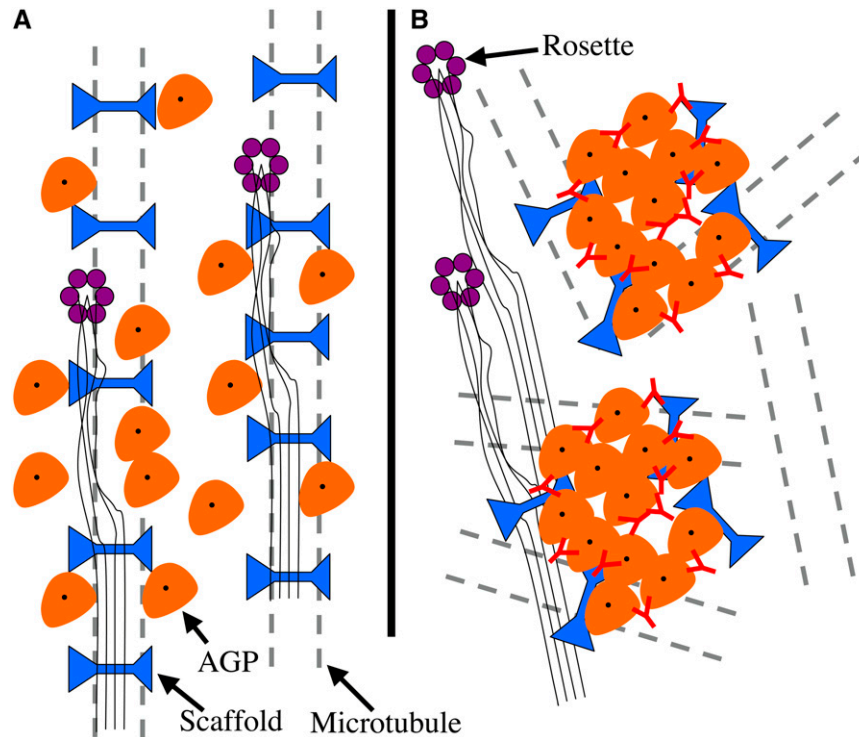


Fig. 3. Depiction of proposed interactions between microtubules, transmembrane scaffold, arabinogalactan proteins (AGP) and cellulose-synthesizing rosettes. The figure is a projection of planes parallel to the plasma membrane, as viewed from the cell wall looking into the cell. (A) Control. Microtubules (dashed lines) orient scaffold proteins (blue), which exist on both sides of the membrane, linked by at least one protein that spans the membrane. The scaffold in turn binds the nascent microfibril (thin, parallel black lines) through its extracellular components. Arabinogalactan proteins (orange) diffuse freely in the plasma membrane, potentially interacting with the scaffold or with the nascent microfibril. (B) Yariv or antibody treatment (red Y's). Arabinogalactan proteins aggregate and derange the scaffold proteins, which in turn disrupt the orientation of cortical microtubules and potentially also cellulose microfibrils. Alignment of cortical microtubules and cellulose microfibrils is known to become uncoupled in the presence of certain compounds (morlin, cobtorin), which might be explained if the compounds target the scaffold.

that arabinogalactan proteins are linked to microtubules physically through intermediary transmembrane proteins. Instead, these elements might be linked through a signaling cascade. Microtubules, although originally viewed as load-bearing struts, are increasingly realized to participate in signal transduction. In *Arabidopsis* roots treated with aluminum or glutamate, cortical microtubules undergo transient depolymerization, which has been argued to be a signaling event analogous to membrane depolarization (Sivaguru et al., 2003). The kinetics of microtubule disruption caused by aluminum are similar to those observed for active Yariv (Nguema-Ona et al., 2007). The key signaling enzyme, phospholipase D, has been shown to bind both microtubules and the plasma membrane (Gardiner et al., 2001). Note, however, that subsequent results implicating phospholipase D and microtubule organization are doubtful because they relied on the inhibitor *n*-butanol, which surprisingly was found to depolymerize purified microtubules in vitro (Hirase et al., 2006). Another key signaling molecule, phospholipase C, is activated in wheat (*Triticum turgidum*) roots by microtubule disorganization or depolymerization, an activation that is important for regulating protoplast volume (Komis et al., 2008).

Cell wall polysaccharides have also traditionally been viewed as structural elements, but arabinogalactan proteins are increasingly found to play informational roles. In plasmolyzed tobacco cells, a green fluorescent protein (GFP)-tagged arabinogalactan protein labels the plasma membrane rather than the cell wall (Sardar et al., 2006), and in *Arabidopsis* roots the same reporter

forms aggregates rapidly following treatment with active Yariv or antibodies (Nguema-Ona et al., 2007). These results suggest that at least some arabinogalactan proteins are not cross-linked into the wall and are able to diffuse within the plasma membrane.

Given that they are free to diffuse, arabinogalactan proteins might interact with receptor kinases and other machinery of signal transduction on the plasma membrane. Evidence of various kinds suggests that such an interaction may occur (Seifert and Roberts, 2007). To cite four examples: first, arabinogalactan proteins are implicated in angiosperm embryogenesis and pattern formation (van Hengel et al., 2001; Rauh and Basile, 2003). Second, treatment with active Yariv for 1 h substantially modifies the transcriptome of *Arabidopsis* tissue culture cells (Guan and Nothnagel, 2004). Third, the *Arabidopsis* mutant, *salt overly sensitive5* (*sos5*), has roots that swell profoundly in the presence of salt (no such swelling occurs in the wild type) and harbors a mutation in a gene for a fasciclin-like arabinogalactan protein (Shi et al., 2003). Finally, a soluble inducer of xylem differentiation is an arabinogalactan protein (Motose et al., 2004). These diverse examples, among others, implicate arabinogalactan proteins in cellular signal transduction pathways.

Once stimulated, an arabinogalactan protein might interact with a plasma membrane receptor, such as a kinase or ion channel. Given the diversity among both arabinogalactan proteins and receptors, we can invoke a diversity of pathways, some of which would lead to a cytosolic calcium signal (Roy et al.,

1999; Pickard and Fujiki, 2005) and others to microtubule disorganization. It would be interesting to know to what extent examples like those cited in the previous paragraph involve disturbance to either or both calcium homeostasis and cortical microtubules.

Finally, if the microtubule disorganization following arabinogalactan-protein binding reflects the output of a signal cascade, then what is the input? Outside of the laboratory, a plant never encounters Yariv reagent. One answer is wounding. When a herbivore bites through a leaf, the distribution of stress within the remaining cell walls will change abruptly, rearranging cell wall polymers, at least to some extent. In view of their mobility, arabinogalactan proteins might be particularly susceptible to stress-induced rearrangement. Supporting this answer are the results of two studies on arabidopsis cell cultures: active Yariv induces programmed cell death (Gao and Showalter, 1999), which often accompanies wounding, and the transcriptome in Yariv-treated cells resembles that of wounded arabidopsis tissues (Guan and Nothnagel, 2004). Assessing the plausibility of this answer will require a better understanding of how stress within the wall is distributed over an organ and how individual cell wall polymers bear this load. Nevertheless, even without identifying specific inputs and outputs, we may visualize arabinogalactan proteins communicating across the plasma membrane to microtubules at the core of a signaling cascade.

Concluding remarks—The function of arabinogalactan proteins in regulating cell morphology was recognized for many years through the use of active Yariv. It has been known for even longer that cortical microtubules are important for cell growth and development. That these components are linked in morphogenesis was suggested by the discovery that both arabinogalactan proteins and cortical microtubules are altered in the abnormally swollen trichoblasts of *reb1* (Andème-Onzighi et al., 2002) and confirmed in studies in which arabinogalactan proteins were disturbed exogenously (Sardar et al., 2006; Nguema-Ona et al., 2007). Thus, there is intercourse between microtubules and arabinogalactan proteins, and the challenge now is to find the wires transmitting the conversation. Attractive candidates include COBRA, phospholipases, and perhaps dynamic nano-domains in the plasma membrane. Eavesdropping on the conversation will reveal essential secrets of plant morphogenesis.

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